EFFECTS OF LIGHT ADAPTATION ON THE PURPLE MEMBRANE STRUCTURE OF *HALOBACTERIUM HALOBIUM*

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ABSTRACT Absorption, circular dichroism and optical rotatory dispersion of the bacteriorhodopsin containing purple membrane from *Halobacterium halobium* were studied in regard to the structural stability of this membrane during the photoisomerization of the retinal of the bacteriorhodopsin from the 13-cis to the all-trans configuration. The following conclusions were reached: (*a*) the macromolecular structure (protein-protein interaction which may result in the possible exciton interaction of the retinal π–π^* (NV_1) transition moments and protein-lipid interaction) are not significantly altered, (*b*) possibilities of delocalized conformation changes of the apoprotein involving secondary and/or tertiary structure can be ruled out, (*c*) localized secondary structure conformation changes of the apoprotein must be limited to the involvement of no more than one or two amino acid residues and localized tertiary structure conformation changes of the apoprotein must be limited to a very short segment of the protein chain containing only a few aromatic amino acid residues, and (*d*) the interaction between the apoprotein and retinal seems to be relatively more pronounced when the retinal is in the all-trans form than the 13-cis form and also the apoprotein seems to impose a more pronounced dissymmetric constraint on the retinal in the all-trans form than in the 13-cis form.

INTRODUCTION

The purple-colored cell membrane of an extremely halophilic bacterium, *Halobacterium halobium*, contains a single protein species which constitutes about 75% of its dry weight and to which retinal is bound by a protonated Schiff base (1). This chromoprotein, termed bacteriorhodopsin by analogy to the visual chromoprotein rhodopsin, may act as a photosensor (2) and a photocoupler involving the transduction of hydrogen ions across the cell membrane (3). The resulting proton gradient may function in ATP synthesis (4) and in active transport (5). The bacteriorhodopsin exists in two relatively stable states: the dark-adapted form absorbing maximally at about 560 nm and the light-adapted form absorbing maximally at about 570 nm. The first determination of the isomeric configuration of the retinal of the dark- and light-adapted states of bacteriorhodopsin indicate a mixture of all-trans and 13-cis retinal and all-trans retinal, respectively (6). In a recent study using the same technique, thin layer chroma-

tography, only 13-cis retinal was found for the dark-adapted state (7). No apparent reason was given for the discrepancy and as yet the discrepancy is unresolved. Light causes isomerization of the 13-cis to the all-trans form during light adaptation of the bacteriorhodopsin. The photocoupling capability of purple membrane is associated with the light-adapted form. In contrast, little is known about the dark-adapted form of the membrane and no physiological function has yet been attributed to it.

Electron micrographs have shown that purple membrane preparations consist of round-to-oval planar sheets of membrane fragments approximately 5,000 Å in diameter (8, 9). A model for the molecular structure of this membrane has emerged from recent X-ray diffraction and electron microscopy studies (8, 10–12). Unique features of this model are the extreme rigidity of the structural organization and the equivalence of the local environments of the bacteriorhodopsins within the membrane. The bacteriorhodopsin molecules are arranged in clusters of three forming a hexagonal lattice. A three-fold axis of symmetry, perpendicular to the plane of the membrane is located at the center of each protein cluster. The protein molecules are fixed rigidly in the membrane lattice and span the entire 45 Å thickness of the membrane. Electron spin resonance measurements have provided further evidence for the extreme rigidity of the structure in which a large proportion of the lipid moiety of the membrane is loosely associated with the protein structure (13). The possibility of intermolecular exciton interaction between the π–π^* (NV_1) transition moments of the retinals, first suggested by this laboratory (14, 15) and later by others (16, 17), provides further evidence for the unique rigidity of this membrane.

The bacteriorhodopsin molecule has a reported molecular weight of 26,000 daltons (1), 20,000 daltons (9, 18) and most recently 25,000 daltons (19) with approximately 12% aromatic amino acid residues and no cysteine (1, 19). X-ray diffraction analysis indicates that the apoprotein consists of seven α-helical polypeptide rods perpendicular to the plane of membrane. The total α-helical content of the protein has been estimated to be about 70–80% based on a polypeptide rod length of 35–40 Å and a molecular weight of 26,000 daltons (10–12).

Conformation change without doubt is one of the most suggested mechanisms for biological function at the molecular level. Although the type of conformational change is usually not implicitly stated, delocalized conformation changes are of greater importance in mechanisms of biological function since they provide the means by which information as mechanical free energy can be transmitted over appreciable distances. In contrast, localized conformation changes are of importance in chemical mechanisms where there is a need for energy localization (20).

In this communication we address ourselves to the problem of the structural stability of purple membrane during light adaptation which results in the 13-cis to all-trans stereoisomerization of the retinal. In specific we ask the following questions: (*a*) Does light adaptation cause a change in the molecular organization of this membrane? (*b*) Does light adaptation induce a delocalization conformation change in the apoprotein of the bacteriorhodopsin? (*c*) Does light adaptation significantly affect the interaction between the retinal and apoprotein? Evidence is presented from absorption,

circular dichroic (CD) and optical rotatory dispersion (ORD) studies of the dark- and light-adapted forms of the purple membrane to rule out delocalized conformation change of the apoprotein during light perturbation. Any changes in the apoprotein's secondary structure must involve no more than one or two residues and in the case of its tertiary structure no more than a few aromatic residues. The evidence also indicates that the integrity of the molecular organization of the membrane is maintained during light adaptation. The only changes observed with light adaptation are those associated with the interaction between the retinal and apoprotein. The dissymmetric constraint imposed on the retinal by the apoprotein in the light-adapted form of the membrane seems to be relatively more pronounced than in the dark-adapted form. Evidently the molecular organization and the protein structure of this membrane is extremely stable to light adaptation which results only in the stereoisomerization of the retinal.

MATERIALS AND METHODS

Cultures of *Halobacterium halobium* R_1 were grown and the purple membrane isolated according to the procedures of Becher and Cassim (9). The isolation procedures yielded a highly purified preparation as determined by electron microscopy, SDS disc gel electrophoresis, and absorption spectroscopy. The criteria for purity has been discussed in a previous publication in detail (9).

Purified purple membrane samples in 0.02 M potassium phosphate buffer (pH 7.0) were prepared under ordinary laboratory light conditions. The samples were then dark-adapted by placing in the dark at 4°C for 48 hr. Further dark-adaptation had no effect on the absorption or CD spectra of the membrane. (No difference in the CD or absorption spectra was found between purple membrane samples prepared in the dark and samples prepared in light and then dark-adapted). Dark-adapted samples were light-adapted by 3-min exposures to intense (500 W DAK projection lamp) light filtered through 3.2 cm of water, 3.2 cm of 2.2% $CuSO_4$ solution, and a n. 12 gelatin Wratten filter. The filter combination selected light from 500 to 700 nm, the wavelength region of maximal absorption of the bacteriorhodopsin. Samples were maintained at 25°C during illumination by employing a specially designed thermostatable cell jacket. Additional light exposure, up to 3 h, had no further effect on the spectra of the membrane samples. Furthermore, the effects of light adaptation on the spectra of the dark-adapted form of the membrane could be repeated after several dark-light adaptation cycles without detectable changes.

Absorption spectra were measured by a Cary Model 118C low UV double-beam recording spectrophotometer with scattered transmission accessory (Varian Instrument Division, Palo Alto, Calif.). The following spectrophotometer constants were maintained during absorption measurements: time constant, 1 s; absorption range, 1.0 from 800 to 350 nm, 2.0 from 350 to 240 nm, 0.5 from 260 to 180 nm; spectral display, 20 nm/in from 800 to 350 nm and 10 nm/in from 350 to 180 nm; recording speed 0.5–1 nm/s from 800 to 350 nm; 0.2 to 0.5 nm/s from 350 to 220 nm, and 0.05 nm/s from 220 to 180 nm. In order to show detail, certain regions of the absorption spectra were enlarged by further adjustment of the recording speed and spectral display. Nitrogen flushing of the spectrophotometer was maintained during measurement of the far ultraviolet spectra to prevent absorption of gaseous oxygen below 200 nm.

CD and ORD spectra were measured by a Cary Model 60 spectropolarimeter equipped with a model 6003 circular dichroism attachment (Varian). The CD mode was calibrated with standard solution of *d*-10-camphorsulphonic acid (Aldrich Chemical Co., Milwaukee, Wis.) by the method of Cassim and Yang (21). The following spectropolarimeter constants were maintained during CD measurements: time constant, 1 s from 625 to 220 nm, 3 s from 220 to 185 nm; range,

0.02 from 625 to 240 nm, 0.04 from 260 to 185 nm; spectral display, 15 nm/in from 625 to 290 nm and 3 nm/in from 300 to 185 nm; recording speed, 5–30 nm/min from 625 to 280 nm and 1–5 nm/min from 300 to 185 nm; photomultiplier voltage, less than 0.5 kV. The ORD was recorded below 260 nm with the following spectropolarimeter constants: time constant, 1 s from 260 to 220 nm and 3 s below 220 nm; range, 0.04; spectral display, 3 nm/in; recording speed, 1–5 nm/min; photomultiplier voltage, less than 0.5 kV.

A constant temperature of 25°C with fluctuations less than 0.1°C was maintained during all spectral measurements. The spectra of a given purple membrane solution was recorded at least twice and in some cases several times. No observable spectral changes were evident indicating that the purple membrane solutions were stable under all recording conditions utilized in this study.

Optically matched pairs of rectangular spectrophotometer cells (Pyrocell Manufacturing Company, Westwood, N.J.) with fused silica windows and path lengths of 1 cm and 1 mm were employed in absorption measurements from 800 to 240 nm and from 260 to 180 nm, respectively. Cylindrical spectrophotometer cells (Pyrocell) with fused silica windows and path lengths of 1 cm and 1 mm were employed in CD measurements from 625 to 240 nm and from 260 to 185 nm, respectively. Cell path lengths were calibrated in a manner previously described in detail (22).

The ORD and the CD in the far ultraviolet wavelength region were recorded for the same samples of purple membrane in the same 1 mm optical cells. The CD spectrum was transformed to ORD by application of the Kronig-Kramers transfer equation. The CD spectrum from 188 to 180 nm was extrapolated to approximate the tail of a gaussian band. Slight variations in the shape of the extrapolated tail of this band do not result in significant changes in the calculated ORD (22). A computer program written in Fortran IV Language by Thiéry (23) was used to evaluate the transform equation. Numerical integrations were carried out over data points at 1 nm intervals. Calculations were carried out with an IBM Systems 370/165 at The Instruction Research Computer Center at The Ohio State University. Details of the computational procedure have previously been given by Cassim and Yang (21, 22).

RESULTS AND DISCUSSION

Visible Spectra

The visible spectra of dark- and light-adapted purple membranes are shown in Fig. 1. The major feature of the dark-adapted preparation is a strong band centered at 559 nm which can be attributed to the π–π^* (NV_1) transition of the chromophore. No inflections at 550 nm or 498 nm due to the major contaminant in purple membrane preparations, a bacterioruberin-containing membrane, are present. After illumination, the 559 nm band undergoes a red shift to 567 nm and a 12% increase in intensity in agreement with previously reported results (6). This is indicative of the photoisomerization of 13-cis retinal to all-trans retinal and accompanying changes in the interaction of the retinal with the apoprotein. In view of the discrepancy in the determination of the isomeric configuration of retinal present in the dark-adapted bacteriorhodopsin (6, 7) it is possible that this state of the bacteriorhodopsin may contain some all-trans retinal as well. However, the structural conclusions reached in this communication are invariant to this possibility. There is no indication of a break in the Schiff base bond between retinal and the apoprotein as observed in bovine rhodopsin. Minor bands in dark-adapted membrane are found at 412, 390, and 370 nm. After illumination, these

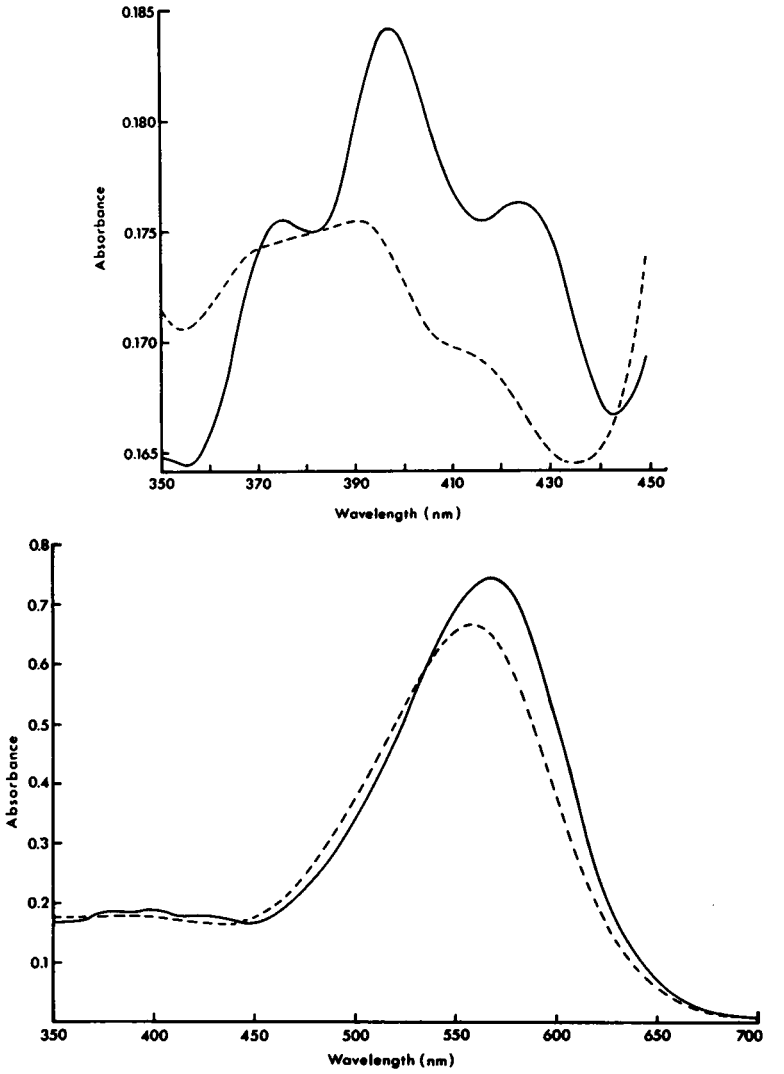

FIGURE 1 Visible absorption spectra of the purple membrane of *H. halobium*. Dashed curve, dark-adapted membrane; solid curve, light-adapted membrane. The optical path length was 1.00 cm.

bands undergo red shifts to 424, 397, and 374, respectively, with 0.5–5.0% increases in intensity. These bands are believed to be associated with the other π–π^* transitions of retinal.

The visible CD spectra of dark- and light-adapted purple membranes are shown in Fig. 2. The major features of the dark-adapted spectrum include negative and positive bands at 595 and 525 nm with a crossover at 563 nm. The intensity of the 595 nm band is 0.8 times that of the 525 nm band. These spectral results are to be contrasted with those obtained for visual rhodopsins in which each visible CD band can be cor-

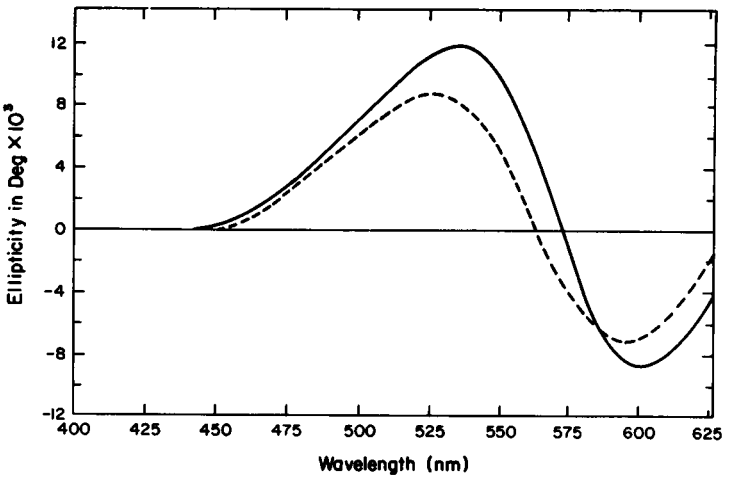

FIGURE 2 Visible CD spectra of the purple membrane of *H. halobium* from 625 to 400 nm. Dashed curve, dark-adapted membrane; solid curve, light-adapted membrane. The absorbance of dark-adapted sample at 559 nm was 1.34 and the optical path length was 1.00 cm.

related with a visible absorption band (24). Illumination of the sample results in a red shift of the bands to 602 and 535 nm with an intensity increase of 25 and 35%, respectively. The crossover shifts to 574 nm.

The positive and negative CD bands are located 34 nm below and 36 nm above the position of the 559 nm absorption band in dark-adapted purple membrane. Furthermore, the CD crossover at 563 nm is located near the wavelength of the absorption band at 559 nm. On illumination, both the absorption and CD bands undergo an intensity increase and red shift, retaining their approximate relative positions. These results may suggest exciton interaction between the retinal π–π^* (NV_1) transition moments of different bacteriorhodopsin molecules (14). However, it is also possible to explain these results in terms of two nearly degenerate transitions with opposite rotatory strengths as suggested by Hudson for certain conjugated polyenes similar to retinal (25). Another possibility may be that there are two different retinal binding sites on each bacteriorhodopsin apoprotein. However, this possibility is inconsistent with retinal:protein molar ratio unless only one site (on the average) is occupied (1).

The molecular exciton model involves the resonance coupling of like molecules in a close, regular array by interaction of their transition charge distributions. As a result, electronic excitation can be considered only in terms of the assembly of interacting molecules and not of individual molecules. Exciton interaction results in the splitting of energy levels associated with the individual molecules. The transition dipole strengths are distributed between these energy levels giving rise to different absorption bands with different intensities, determined by the geometric arrangements of the interacting molecules and their transition dipole strengths. If the interacting molecules are optically active, then each of the resulting exciton absorption bands will also have a characteristic CD band determined by the geometric arrangement of their interacting

molecules and their transition dipole strengths. Since the total exciton contribution to the rotational strength must be zero by the sum rule, CD exciton bands must have positive and negative signs.

In the case of purple membrane, the positive and negative CD bands could be interpreted as a result of exciton interaction between the chromophoric π–π^* (NV_1) transition moments responsible for the absorption band centered between the CD bands. Although exciton interaction would also be expected to give rise to splitting of the chromophoric π–π^* (NV_1) absorption band (for cyclic trimer two exciton bands are possible with one being doubly degenerate), the resulting bands could apparently overlap sufficiently to appear as the broad single band as seen in Fig. 1. The nonconservative profile of the CD spectrum due to the nonzero sum of the rotational strengths could be attributed to an additional (non-exciton) positive CD band arising from the induced dissymmetry of the chromophore in the environment of the apoprotein. Calculations employing the molecular exciton model (26) and based on the arrangement of bacteriorhodopsin molecules in clusters of three with a three-fold axis of symmetry (cyclic trimer) have been made. Computations were carried out with a optimization curve fitting program written in Fortran IV. Details will be published elsewhere (T. Hsiao, D. D. Muccio, and J. Y. Cassim, manuscript in preparation). These computations demonstrated that exciton interaction between the three retinals, together with a positive non-exciton contribution from the induced retinal optical activity, could account for the visible CD and absorption spectra of the purple membrane. It was also demonstrated that this could be achieved with retinal positions in the cluster being within those suggested by recent X-ray diffraction and electron microscopy studies (10–12).

Further support for the exciton interpretation of the purple membrane visible spectra is obtained from two recent studies. Solubilization of the membrane by Triton X-100, which causes a disruption of the membrane structure, results in a single positive CD band and only a 5% loss in absorption (16). Successive additions of retinal to the apomembrane result in, at low levels of regeneration as indicated by the absorption spectra, a majority of the three-site clusters containing only one retinal (17). As a result, little exciton contribution to the CD spectra is expected and, in fact, the resulting CD is dominated by a single positive band. It is much more difficult to reconcile these results in terms of a model based on two nearly degenerate transitions with opposite rotatory strengths or a model based on two different retinal binding sites than one based on exciton interaction.

If the exciton interaction is a reasonable explanation for the unique visible CD of this membrane then the fact that there is no significant change in the shape of the CD curve upon light adaptation strongly suggests that the rigid molecular arrangement of the membrane remains invariant during the photostereoisomerization of the retinal. Exciton interactions among chromophores are extremely sensitive to the spatial arrangement of the interaction chromophores (26). Any change in the relative positions and orientations of the proteins and/or retinals within the protein clusters would be reflected in the visible CD spectrum. The magnitude and position of the main ab-

sorption band of the retinal (attributed to the π–π^* (NV_1) transition) in rhodopsin is strongly dependent on the configuration of the retinal and its interaction with the apoprotein. A wide variation in the wavelength position of this band has been observed for various rhodopsins (27, 28). Also in solution the oscillator strength of the main band of the all-trans retinal is greater than that of the 13-cis retinal (29). In view of these facts the red shift and the intensity increase of the 559 nm absorption band upon light adaptation can be attributed to modifications of the interaction between the retinal and the apoprotein resulting from the 13-cis to all-trans stereoisomerization. The changes in the CD spectra can be explained similarly. However, a part of the increase in the absolute ellipticity should be attributed to an increase of the dissymmetric constraints imposed on the retinal by the apoprotein. More convincing arguments in support of this statement are presented below from the effects of light adaptation on the negative CD band at 317 nm.

Near Ultraviolet Spectra

The near UV absorption spectra of dark- and light-adapted purple membrane are shown in Fig. 3. Bands are centered at 290, 280, and 274 nm in the dark-adapted membrane spectrum with approximately 1.3, 1.7, and 1.7 times the intensity of the 559 nm band, respectively. With light exposure, the absorption spectrum undergoes a very small decrease in intensity from 350 to 300 nm and a very small increase in intensity from 300 to 260 nm. Inflections at 340 and 323 nm are found in dark-adapted purple membrane which can be attributed to the weak π–π^* transitions of 13-cis retinal. On illumination and isomerization of the retinal to the all-trans configuration, these inflections are red shifted to 349 and 325 nm with about 8% decrease in intensity. Inflections at 283, 275, 269, 264, and 258 nm are also apparent. The bands and inflections in the 295–250 nm spectra can be primarily attributed to the π–π^* transitions of the amino acids tryptophan, tyrosine, and phenylalanine. The distinct band at 290 nm indicates a significant contribution from tryptophan. No absorption is attributed to the disulfide bond of cysteine since this amino acid is not found in bacteriorhodopsin. With light exposure, these bands and inflections do not undergo shifts in wavelengths. The intensity of the 290 nm band increases by 0.5–1.0% and the intensities of the 283, 286, and 275 nm inflections increase about 2% with lesser increases in the remaining inflections.

The CD spectrum of dark-adapted purple membrane from 380 to 290 nm is shown in Fig. 4. The main feature of this spectrum is an intense band centered at 317 nm with 1.6 times the intensity of the 525 nm CD band of dark-adapted membrane. After illumination, the band undergoes a 25% increase in intensity with no shift in wavelength. This intense CD band is particularly interesting because the transition(s) giving rise to this band apparently are magnetic-dipole allowed but electric-dipole forbidden since only absorption bands with weak oscillatory strengths are found in this wavelength region (Fig. 3). Therefore, no significant splitting of this band by exciton interaction is expected and none is observed (Fig. 4). Also, since the retinal is not intrinsically optically active this suggests that the apoprotein of the bacteriorhodopsin

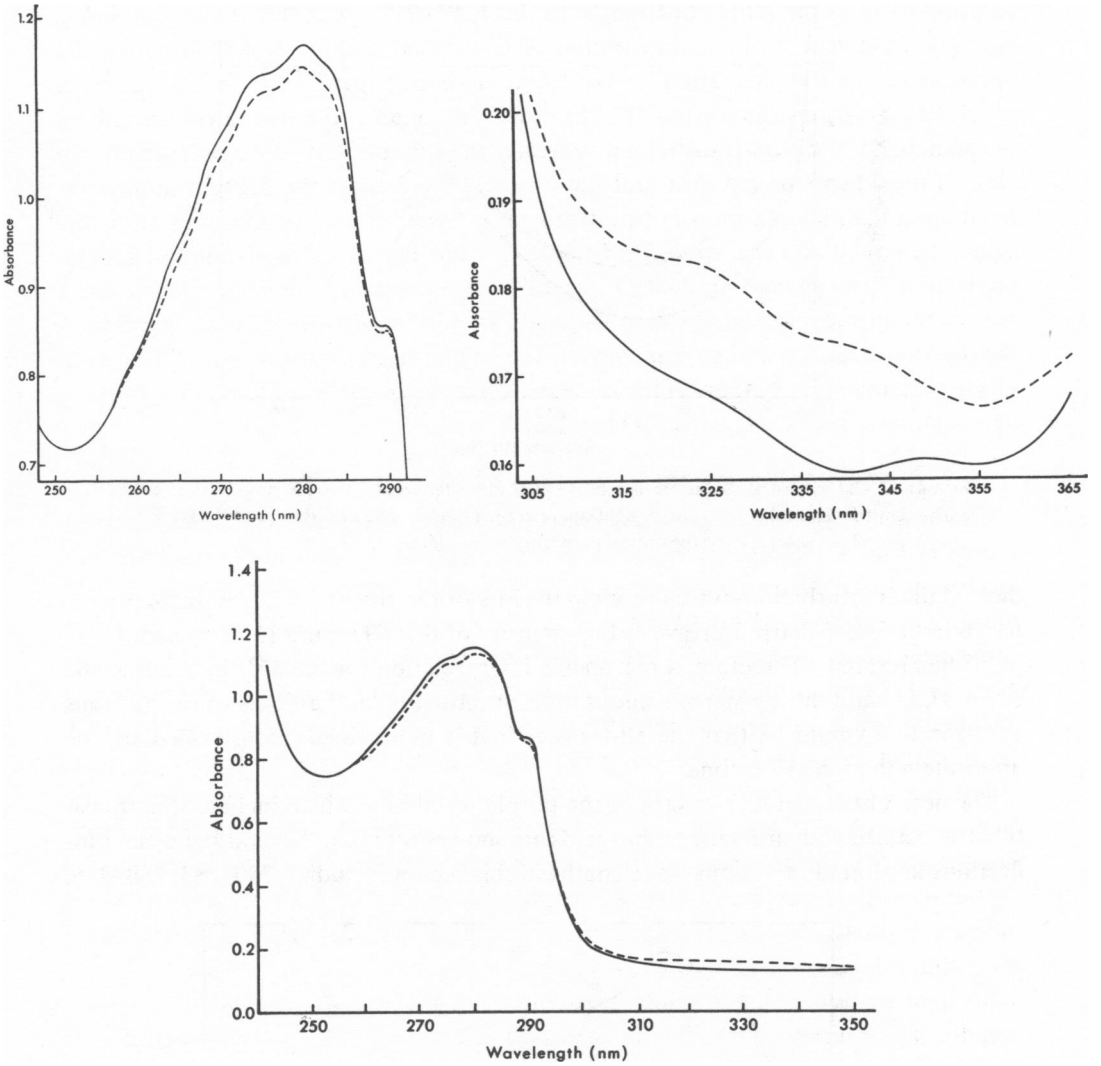

FIGURE 3 Near ultraviolet absorption spectra of the purple membrane of *H. halobium*. Dashed curve, dark-adapted membrane; solid curve, light-adapted membrane. The absorbance of dark-adapted sample at 559 nm was 0.67 and the optical path length was 1.00 cm.

must impose exceptionally strong dissymmetric constraints on the retinal to induce such a high rotatory strength. Of course, the possibility of exciton interaction between the π–π^* (NV_1) transition moments of the retinals is also indicative of the rigidity of the retinal-apoprotein secondary non-covalent bond. In view of the behavior of this CD band to light adaptation of the membrane as shown in Fig. 4 the intensity of this band may be a sensitive indicator of the rotational freedom of the retinal molecule relative to the apoprotein molecule. Recent studies in this laboratory on the effects of detergents and select reagents on the structural stability of this membrane have in-

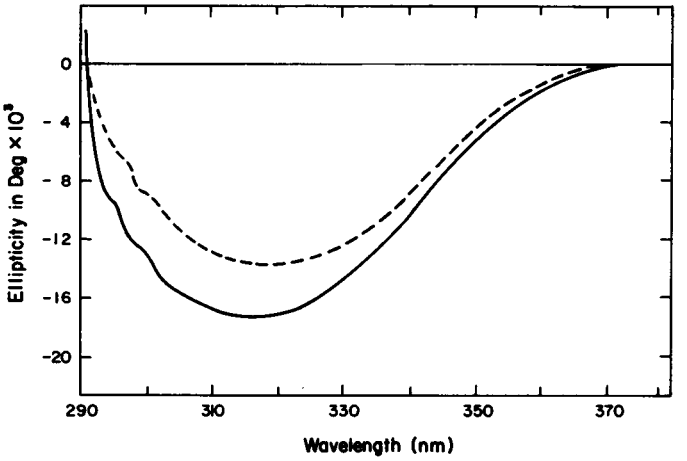

FIGURE 4 CD spectra of the purple membrane of *H. halobium* from 380 to 290 nm. Dashed curve, dark-adapted membrane; solid curve, light-adapted membrane. The absorbance of dark-adapted sample at 559 nm was 1.34 and the optical path length was 1.00 cm.

dicated that perturbations which result in the loss of the rigidity of the retinal-apoprotein bond cause a sharp decrease in the intensity of this CD band (J. Y. Cassim, unpublished results). Therefore, a reasonable interpretation for the 25% increase of the 317 nm CD band during stereoisomerization of retinal from the 13-cis to the all-trans configuration would be that the all-trans retinal is more rigidly constrained by the apoprotein than the 13-cis one.

The near ultraviolet CD spectra of the purple membrane which includes the transitions associated with aromatic amino acids are shown in Fig. 5. Several bands and inflections are found at various wavelengths in this region including 290, 283, 280, 275,

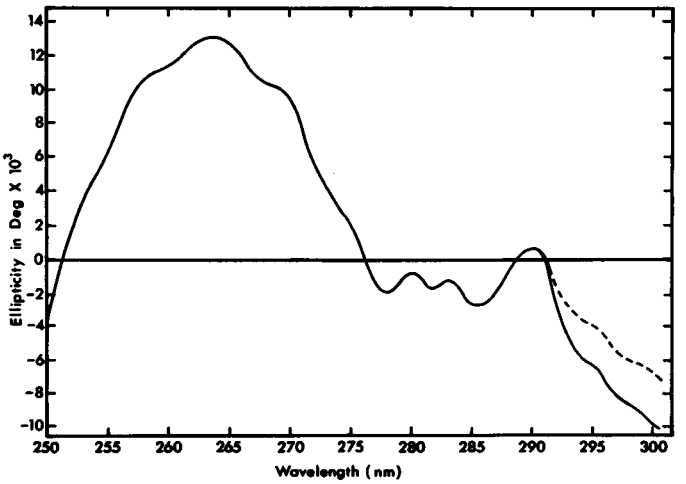

FIGURE 5 Near ultraviolet CD spectra of the purple membrane of *H. halobium*. Dashed curve, dark-adapted membrane; solid curve, light-adapted membrane. The absorbance of dark-adapted sample at 559 nm was 1.34 and the optical path length was 1.00 cm.

269, 264, and 258 nm. In every instance these CD features are within 1 nm of bands or inflections found in the absorption spectra. The major features of the CD spectra include a positive band at 290 nm which suppresses the broad negative tail of the 317 nm band. Other major features are intense, positive bands at 269, 264, and 258 nm. The 264 band, which is 1.5 times the intensity of the 525 nm band in dark-adapted purple membrane, has a mean molar residue ellipticity of 277 deg · cm^2/dmol based on an average residue molecular weight of 103 daltons, a molecular weight of 25,000 daltons for bacteriorhodopsin (19) and an extinction coefficient of 63 $mmol^{-1} \cdot cm^{-1}$ for light-adapted purple membrane at 568 nm (30). The intensity of the near ultraviolet CD spectrum, considering the percentage of aromatic amino acids in the protein (12%), is comparable to that of other specific proteins studied (31, 32).

On light adaptation of the purple membrane, no measurable change in the near ultraviolet CD of purple membrane is found. Due to the relatively high oscillatory strengths and low rotatory strengths of the transitions from 300 to 250 nm, a low signal-to-noise ratio is inherent in the CD measurements of the purple membrane in this region. Consequently, it is estimated that changes of up to 5% in the ellipticity at 260 nm could occur without detection. The significances of the near ultraviolet CD studies in regards to conformation change are discussed together with those of far ultraviolet spectra in the next section.

FAR ULTRAVIOLET SPECTRA

The far UV absorption spectrum of the dark-adapted purple membrane is shown in Fig. 6. The spectrum resembles previously published spectra of proteins with appre-

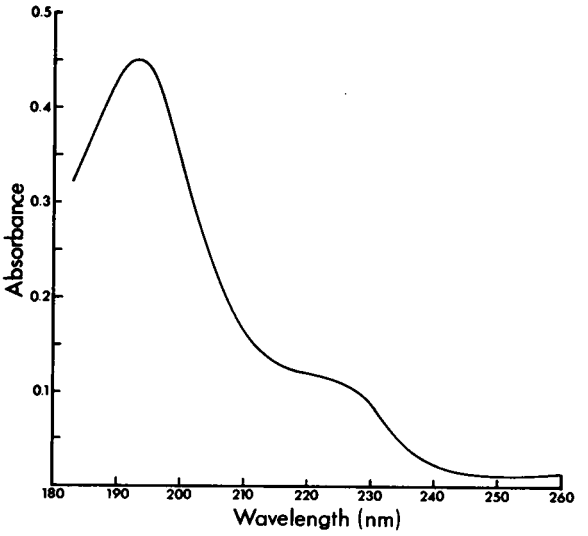

FIGURE 6 Far ultraviolet absorption spectrum of the purple membrane of *H. halobium*. The spectra of dark-adapted and light-adapted membrane are identical within an experimental uncertainty of about 0.05% at 194 nm. The absorbance of dark-adapted sample at 559 nm was 0.096 and the optical path length was 1.04 mm.

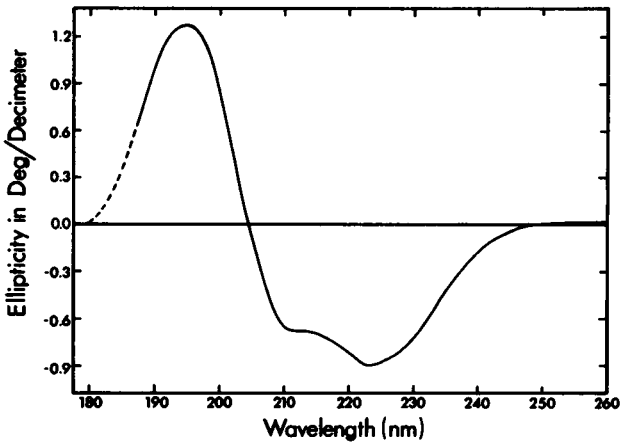

FIGURE 7 Far ultraviolet CD spectrum of the purple membrane of *H. halobium*. The spectra of dark-adapted and light-adapted membrane are identical within an experimental uncertainty of about 1% at the 223 nm band. The absorbance of dark-adapted sample at 559 nm was 0.13 and the optical path length was 1.00 mm. The dashed line represents extrapolation of the positive ellipticity into the inaccessible spectral regions below 188 nm. See text for details.

ciable amounts of α-helical secondary structure (33). The major features of the spectrum are a shoulder at 225 nm which can be attributed to the amide n–π^* transition and a peak at 194 nm which can be attributed to the perpendicularly polarized component of the Davidov splitting of the amide π–π^* (NV_1) transition by exciton interaction. The 3 to 4 nm red shift of these features relative to the spectra of α-helical polypeptides can be attributed to optical artifacts arising from the particulate nature of the membrane suspension and to the existence of conformations in the protein other than the α-helix. No observable change in the spectrum occurs with light adaptation of the membrane within an experimental uncertainty of 0.05% at the 194 nm peak.

The far ultraviolet CD spectrum of the purple membrane is reproduced in Fig. 7. The two negative bands at 223 and 211 nm are attributed to the amide n–π^* transition and parallel polarized component of the Davidov splitting of the amide π–π^* (NV_1) transition by exciton interaction, respectively. The positive band at 194 nm is attributed to the perpendicularly polarized component of the Davidov splitting. The mean molar residue ellipticities of the bands at 223, 211, and 194 nm are −15,900, −11,900, and 22,300 deg cm^2/dmol, respectively, based on a residue molecular weight of 103 daltons, a molecular weight of 25,000 daltons, and an extinction coefficient of 63 $mmol^{-1} \cdot cm^{-1}$ for bacteriorhodopsin at 568 nm. Although this spectrum resembles, in gross aspect, those spectra previously obtained for partially helical model polypeptides, the intensities of the 211 and 194 nm bands are depressed relative to that of the 223 nm band and all the band maxima have undergone red shifts. Similar deviations have been observed in the CD spectra of several other membranes studied and have been attributed to optical artifacts arising from the particulate nature of the membrane suspensions (34–37). However, it has been pointed out that the particulate artifacts are small

near the 222 nm band, therefore, estimates of the helical content of membrane proteins based on the ellipticity at this wavelength are affected little by these artifacts (38).

Over the past decade the ellipticities of proteins at 222 nm have been used to estimate their helical content. There has been disagreement regarding the proper values for the helix and the random-coil. Recently serious doubts have been raised about the value of such calculations (39). However, we feel that if absolute accuracy is not essential these calculations are still useful in that they provide the only fast means for making rough comparisons of the helical contents of proteins. The most recent published "standard" values for the ellipticities of the α-helix and random-coil forms, based on the parameters of crystallographically determined proteins, are $-32{,}600$ and $-2{,}340$ deg cm^2/dmol, respectively (40). Using these values we estimate the helical content of the bacteriorhodopsin in the membrane to be about 45%. This estimate is in reasonable agreement with the estimates based on X-ray diffraction analysis and electron microscopy, 70–80%, considering the difficulties inherent in the various methods (10–12). Bovine rhodopsin *in situ* in the rod outer segment disk membranes yielded a helical content of about 29% by the same method of estimation (C. Rafferty and J. Y. Cassim, manuscript in preparation). For comparison, the helical contents of non-photosensitive membrane proteins *in situ* were also estimated by this procedure utilizing previously published membrane CD parameters (35, 41–43). In all cases for which CD data was available the helical content was less than those obtained for the bacteriorhodopsin *in situ*. (For example, red blood cell ghosts, 36%, mitochondria, 28%, plasma membrane, 25%, sarcotubular vesicles, 11%, and axonal membrane, 19%). These results are expected in view of the emerging picture of an extremely rigid structural organization for the purple membrane in contrast to other membranes in which there is evidence of structural fluidity. α-helices are usually found in biological structure where rigidity, strength, and stability are essential. The important question is, of course, what purpose this rigidity serves in the function of this membrane? One may speculate that this rigidity provides the proper environment for exciton interaction which would increase the efficiency of light absorption by the retinal due to the delocalization of excitation over the protein clusters.

The far ultraviolet CD of dark- and light-adapted purple membranes are indistinguishable. In the neighborhood of the 223 nm band, the reproducibility of the spectra was about 1% of the ellipticity at 223 nm. This 1% uncertainty in ellipticity is equivalent to a helical content uncertainty of 0.6% in this case. Considering that the bacteriorhodopsin apoprotein is composed of about 243 amino acid residues (19), then the smallest change which would be detected in the CD spectra corresponds to a net change in participation of approximately one to two amino acid residues in the helical structure of the apoprotein upon stereoisomerization. Let us now consider what this lack of observable far and near ultraviolet CD spectral changes during photostereoisomerization means in respect to the apoprotein's structural stability. The interpretation of such spectral results in regard to protein structural changes during perturbation has been discussed in length in a recent publication from this laboratory (20). Information concerning the secondary structures of proteins are contained both

in the far and near ultraviolet CD spectra of the proteins in solution. However, since the spectra in these two regions are due to different π–π^* transitions, amide and aromatic, they provide two independent probes of secondary structure. The fact that both probes are not perturbed by photostereoisomerization strongly suggests not only that there has been no net secondary structure change within experimental uncertainty but also that the possibility of compensating secondary structural changes can be ruled out. The experimental uncertainty sets the upper limits for possible secondary structure changes. In this case the involvement of no more than two amino acids in possible helical content change is the upper limit. That is, if there is a secondary structure change it must be highly localized in a very small section of the protein chain. The fact that the near ultraviolet absorption spectrum is invariant to photostereoisomerization provides further support for this conclusion.

The near ultraviolet CD spectra also provides information about possible changes in the tertiary and quaternary structure of proteins (31, 32). The CD spectra in this region has been attributed to the dissymmetric interactions of the side chains of the aromatic residues and cysteine with the protein's local environments. Therefore, this spectrum is highly sensitive to any conformational change (secondary, tertiary, or quaternary) which alters these local environments. Bacteriorhodopsin apoprotein contains 30 aromatic residues and no cysteine out of a total amino acid content of 243 (19). If these aromatic residues are randomly distributed along the protein chain, then any conformational distortion transmitted over a portion of the chain containing several of these residues should alter their local environments sufficiently to cause a change in the CD spectrum. However, if the conformational distortion is limited to a very short portion of the chain, then this might not sufficiently alter the environment of enough aromatic residues to produce a measurable change in the CD spectrum which is a sum of the contributions from all such residues. Since the near ultraviolet CD of bacteriorhodopsin did not change within a 5% experimental uncertainty during photostereoisomerization, it is evident that conformation change due to tertiary structure alteration could not have been delocalized over a large segment of the protein chain. That is, any conformation change involving the secondary and/or tertiary structure of the apoprotein resulting from photostereoisomerization must be highly localized at the site of the interaction of the retinal and the apoprotein and the resulting conformation distortion does not spread throughout the apoprotein. Although the absorption spectrum in this region undergoes small (0.5–2.0%) increases in intensity on photostereoisomerization, these results are in accord with the CD spectral results. These small changes can be attributed to the difference in the absorption of the two retinal stereoisomers and to conformation changes highly localized in the retinal-apoprotein interaction site region. One must bear in mind that protein interactions with any perturbant must involve some kind of conformation change in view of the all encompassing definition of conformation change (44). The important point is not whether a conformation change has occurred but the type of conformation that has occurred. Local conformation changes are inherent in every protein interaction, whereas delocalized conformation changes are unique since they provide the means by

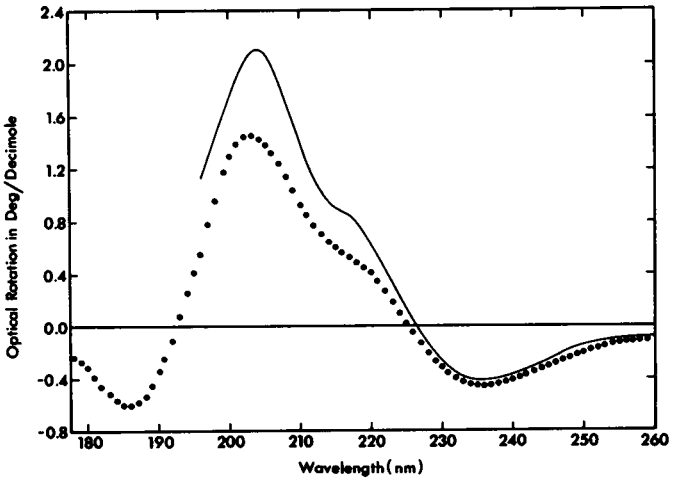

FIGURE 8 Experimental and calculated far ultraviolet ORD spectra of the purple membrane of *H. halobium*. Experimental ORD spectra of dark-adapted and light-adapted purple membrane (solid curve) are identical within an experimental uncertainty of about 3% at the 204 nm peak. Calculated ORD spectrum of dark-adapted or light-adapted purple membrane (solid points) obtained by application of Kronig-Kramers transform to the CD data of Fig. 7. The absorption of dark-adapted sample at 559 nm was 0.148 and the optical path length was 1.00 mm. See text for details.

which information as mechanical free energy can be transferred intra- and intermacromolecularly in biological structures.

The ORD of purple membrane was recorded from 260 to 198 nm (Fig. 8). The spectrum is typical for a protein of relatively high helical content. An intense positive peak at 204 nm (with an inflection at 217 nm) and a negative trough at 235 nm with a crossover at 227 nm are apparent. These features are red shifted in the membrane samples as the result of spectral artifacts associated with particulate systems. No measurable difference between the ORD of light-exposed and dark-adapted purple membrane was found within an experimental uncertainty of 3% at the 204 nm peak.

Studies were also undertaken to determine if the experimental ORD of purple membrane originates entirely from the electronic transitions that give rise to the CD spectrum in the far ultraviolet wavelength region. The same samples of purple membrane in the same optical cells were employed in recording the experimental ORD spectrum (Fig. 8) and the experimental CD spectrum (Fig. 7). The ORD calculated from the experimental CD spectrum (Fig. 8 solid points) by use of the Kronig-Kramers transform integral was compared with the experimental ORD. The 204 nm peak of the experimental curve is found at 203 nm in the calculated curve with a 30% less rotation. A difference curve resembling the tail of one or more positive Cotten effects is obtained by subtracting the calculated curve from the experimental one. This indicates the existence of strong optically active transition(s) just below 185 nm.

The source of the optically active transitions below 185 nm is of interest. Cassim

and Yang (22) demonstrated that the experimental ORD of several synthetic helical polypeptides in the far ultraviolet wavelength range (260–185 nm) can be completely accounted for by the CD spectrum in this wavelength region to within the limits of experimental error by use of the Kronig-Kramers transform integral. These results demonstrate that vacuum ultraviolet optically active transitions below 185 nm, which have been observed experimentally under very special conditions (45), do not contribute significantly to the experimental ORD of synthetic helical polypeptides in the far ultraviolet region. Similar results have also been obtained for polypeptides in the random-coil form (46). Furthermore, analogous studies of several proteins both globular and fibrous have shown a difference between experimental and calculated ORD of no greater than 7% at the 198 nm ORD peak, with the majority of the proteins showing no measurable difference (J. Y. Cassim, unpublished results). Consequently, based on evidence from extensive studies of proteins and synthetic helical polypeptides, the major (30%) difference between the calculated and experimental ORD of purple membrane cannot be largely attributed to protein transitions below 185 nm. This difference also cannot be attributed to optically active transitions of the cysteine disulfide bond since this amino acid is not found in the purple membrane. However, since electronic transitions below 190 nm have been associated with lipids, the differences between the calculated and experimental far ultraviolet ORD may be the result of transition(s) of the lipid component of the purple membrane.

The possibility of the transitions of the lipids strongly contributing to the far ultraviolet ORD of the purple membranes is of particular interest since the purple membrane has been reported to consist of only 25% lipid (1). Optical activity of the lipids could be associated with intrinsic dissymmetry and/or induced dissymmetry of the lipid molecules. Since the purple membrane possess a highly rigid structure in which a large proportion of the lipid is tightly bound to the protein (13), the structure could give rise to the dissymmetric constraints on the lipid which are required for induced optical activity. The fact that the photostereoisomerization of the bacteriorhodopsin does not result in an observable ORD change would be an indication of the stability of the protein-lipid interaction if there optically active transitions below 185 nm can be largely attributed to the lipid transitions.

To summarize, the evidence presented strongly attests to the structural stability of the purple membrane during photoisomerization. The protein-protein interaction which may result in the possible exciton interaction of the retinal π–π^* (NV_1) transition moments and the protein-lipid interaction are not observably altered during this photoprocess. Delocalized conformation changes of the apoprotein involving secondary and/or tertiary structure can be ruled out. Possible localized conformation changes involving secondary structure must be limited to involvement of no more than one or two amino acid residues and in the case of tertiary structure to short segments of the protein chain involving no more than a few aromatic amino acid residues. The only significant structural change during this photoprocess involves the retinal-apoprotein secondary non-covalent linkage. The interaction between the retinal and apoprotein seems to be relatively more pronounced in the light-adapted form of the

membrane and also the dissymmetric constraint imposed on the retinal by the apoprotein seems to be relatively greater. This indicates that the all-trans isomer of retinal is possibly more rigidly held by the apoprotein than the 13-cis isomer.

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